Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1073–1077, February 1982
Biochemistry

Expression of a cloned adenovirus gene is inhibited by *in vitro* methylation†

(microinjection of oocytes from *Xenopus laevis*/*Hpa* II DNA methyltransferase/stability of *in vitro* methylation patterns/gene for adenovirus DNA-binding protein/nuclease S1 analysis of viral RNA synthesized in oocytes)

LILY VARDIMON‡, ARMIN KRESSMANN§, HOWARD CEDAR¶, MARCO MAECHLER§, AND WALTER DOERFLER‡‖

‡Institute of Genetics, University of Cologne, Weyertal 121, 5000 Cologne, Federal Republic of Germany; §Department of Molecular Biology, University of Zürich, Zürich, Switzerland; and ¶Department of Cellular Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel

Communicated by James E. Darnell, Jr., November 2, 1981

ABSTRACT **In many viral and nonviral eukaryotic systems, an inverse correlation has been observed between the extent of DNA methylation at 5′-C-C-G-G-3′ sites and the extent of expression of specific genes as mRNA. The E2a region of adenovirus serotype 2 (Ad2) DNA encodes the Ad2-specific DNA-binding protein required for viral DNA replication. In three lines of Ad2-transformed hamster cells (HE1, HE2, and HE3), multiple copies of the major part of the Ad2 genome persist in an integrated state. Cell lines HE2 and HE3 do not express the DNA-binding protein whereas line HE1 does so. It has been shown that, in cell line HE1, all 5′-C-C-G-G-3′ (*Hpa* II/*Msp* I) sites in the E2a region remain unmethylated. Conversely, in lines HE2 and HE3 lacking expression of the E2a region all *Hpa* II sites are methylated. The cloned E2a region of Ad2 DNA, the *Hin*dIII A fragment in pBR322, was methylated *in vitro* by using *Hpa* II DNA methyltransferase (5′-C-C*G-G-3′) or was left unmethylated. *In vitro* methylation did not break or nick supercoiled circular DNA. Methylated or unmethylated DNA was then microinjected into the nuclei of *Xenopus laevis* oocytes, and the subsequent synthesis of Ad2-specific RNA was monitored. *In vitro*-methylated DNA remained in the methylated state for 24 hr on microinjection into nuclei of *Xenopus* oocytes; unmethylated DNA remained unmethylated. When the injected DNA had been methylated by using *Hpa* II DNA methyltransferase, Ad2-specific RNA was not synthesized as late as 24 hr after microinjection. Unmethylated DNA was readily expressed into Ad2-specific RNA. As an internal control, unmethylated histone genes (h22 DNA) from sea urchin were microinjected together with methylated E2a DNA from Ad2. Ad2-specific RNA was not found; h22 DNA-specific RNA was readily detected. This finding ruled out nonspecific inhibitory effects in the methylated DNA preparation. It was also shown that transcription of the unmethylated *Hin*dIII A fragment of Ad2 DNA in *Xenopus* oocytes was initiated on the late promoter of the E2a region. The same promoter was used in productively infected KB cells. Methylation by *Bsu*RI methylase (5′-G-G-C*C-3′) did not inactivate the *Hin*dIII A fragment. These results provide evidence for the notion that methylated sequences at highly specific sites are involved in the regulation of gene expression. The actual nature of the regulatory signal is not yet understood.**

Evidence has accumulated implicating DNA methylation as a regulatory signal in eukaryotic gene expression (1–5). Two basic approaches have led to the hypothesis that high levels of DNA methylation are associated with the absence of gene expression. (*i*) An inverse correlation has been established between the extent of DNA methylation and the level at which certain genes are expressed as mRNAs for several viral systems (6–13) and a number of eukaryotic genes (14–20). (*ii*) Incorporation of the cytidine analogue 5-azacytidine into the DNA of growing mouse cells results in differentiation of a low percentage of these cells to functional muscle cells (21, 22). Chicken cells can be activated by transient exposure to 5-azacytidine to express endogenous retroviral genes (13).

In this communication, we show that, within 24 hr after microinjection of the gene for the DNA-binding protein (DBP) of adenovirus serotype 2 (Ad2) inserted in pBR322 into nuclei of oocytes from *Xenopus laevis*, viral-specific RNA could be isolated from the oocytes. The Ad2-specific RNA synthesized in *Xenopus laevis* oocytes was shown to be initiated at the late promoter of the E2a region of Ad2 DNA. When the same Ad2 gene was methylated to completion by *Hpa* II DNA methyltransferase (recognition sequence, 5′-C-C-G-G-3′), no Ad2-specific RNA could be detected. These data show that DNA methylation is involved in the control of gene expression and argue against the idea that DNA methylation may have arisen as a consequence of gene inactivation.

MATERIALS AND METHODS

Viral DNA and a Cloned DNA Fragment: Nick Translation. Viral DNA was extracted from purified virions as outlined (23, 24). From the cloned *Hin*dIII A fragment of Ad2 DNA, a fragment comprising the map coordinates 70.7 (*Eco*RI site)–72.8 (*Hin*dIII site) of Ad2 DNA was subcloned. The plasmid was cleaved with *Eco*RI/*Hin*dIII. The DNA of plasmid pBR322 (25) was cut with the same enzymes. The cleavage products were mixed and ligated as described (26). By using standard selection procedures, a clone containing the 70.7–72.8 map-unit fragment was selected. The subcloned fragment carried one of the late promoter leader regions of the E2a segment of Ad2 DNA (27, 28). The histone genes from sea urchin, h22 DNA, were cloned in pBR322 (29). Prior to injection, the DNA fragment containing the histone genes was excised from the plasmid with *Hin*dIII, purified by gel electrophoresis, and recircularized as described (30). For hybridization experiments, all DNA preparations were ^{32}P-labeled by the nick-translation procedure (31) described earlier (32).

For nuclease S1 mapping experiments, a viral DNA fragment comprising the stretch from 71.4 (*Kpn* I site) to 72.8 (*Hin*dIII site) map units of Ad2 DNA was prepared (see Fig. 4*b*). The subclone containing the 70.7–72.8 map-unit fragment of Ad2

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Ad2, adenovirus serotype 2; DBP, DNA-binding protein.

† These results were presented in part at the Spring Meeting of the Institute of Genetics in Cologne, 4-7 March 1981: DNA Methylation and Genome Organization.

‖ To whom reprint requests should be addressed.

DNA (Fig. 1) in pBR322 was cleaved with *Kpn* I/*Hin*dIII. The 493-base-pair fragment generated was purified by electrophoresis on a 4% polyacrylamide gel followed by thermoelution.

***In Vitro* Methylation of a Cloned Ad2 DNA Fragment.** DNA methyltransferase from Hemophilus para-influenzae was purified as described (33, 34). For the reaction with *Hpa* II DNA methyltransferase, DNA at 50 μg/ml was incubated with a saturating amount of enzyme in 50 mM Tris·HCl, pH 7.9/5 mM dithiothreitol/5 μM *S*-adenosyl-L-methionine at 37°C for 1 hr. Then, the solution was adjusted to 0.4 M NaCl/1 mM EDTA/0.2% $NaDodSO_4$ and extracted once with Tris·HCl-saturated phenol and once with chloroform/isoamyl alcohol, 24:1 (vol/vol). The DNA was precipitated from the aqueous phase by ethanol. The *in vitro*-methylated DNA was suspended in injection buffer (10 mM Tris·HCl, pH 7.5/80 mM NaCl) to 250 μg/ml. Unmethylated control DNA was treated in an identical fashion, except that DNA methyltransferase was not added to the reaction mixture. DNA preparations were tested for completeness of reaction by cleavage with *Hpa* II or *Msp* I (35). DNA fragments were analyzed by electrophoresis on horizontal 1.5% agarose slab gels.

Injection of DNA into Nuclei of *Xenopus laevis* Oocytes. The method has been described (36). Five nanograms of DNA in a total volume of 20 nl were injected per oocyte.

Extraction and Analysis of DNA and RNA from *Xenopus laevis* Oocytes: Extraction of RNA from Ad2-Infected KB Cells. The methods have been described (36). In principle, the techniques used were similar to those described for transformed cells (6, 37, 38). DNA preparations extracted from microinjected *Xenopus* oocytes were treated with RNase at 20 μg/ml prior to restriction endonuclease cleavage, slab gel electrophoresis, and Southern blotting (6, 37, 38, 39). Each slot was loaded with the total DNA of two oocytes, comprising 88 pg of cellular DNA and 10 ng of microinjected DNA.

On the other hand, RNA extracted from *Xenopus* oocytes was treated with DNase (50 μg/ml) or was left untreated. The DNase used had been incubated with Na iodoacetate as described (40). RNA preparations (20 μg per slot, the equivalent of 4 or 5 oocytes) were analyzed by electrophoresis on 0.8% agarose slab gels containing 2.2 M formaldehyde, by blotting and DNA·RNA hybridization as described (38). In DNA·DNA or DNA·RNA hybridization experiments, DNA preparations were used as probes that had been ^{32}P labeled by nick-translation (31). Ad2 DNA, h22 DNA excised from pBR322, and pBR322 DNA alone were used as probes.

Analysis of the Ad2-Specific RNA from *Xenopus laevis* Oocytes and from Ad2-Infected KB Cells by Using the Single-Strand-Specific Nuclease S1. Was the Ad2-specific RNA synthesized in *Xenopus* oocytes initiated on the late promoter/leader of the E2a region (27, 28)? The following experiment was performed (41). Unlabeled total RNA from normal or microinjected oocytes or from nuclear or cytoplasmic RNA from Ad2-infected KB cells was hybridized to the leftward-transcribed-strand of the 71.4 (*Kpn* I site)–72.8 (*Hin*dIII site) map-unit fragment of Ad2 DNA (see above and Fig. 4) at 68°C for 2 hr in 1 M NaCl/0.01 M Tris·HCl, pH 7.5/1 mM EDTA. The viral DNA fragment was ^{32}P labeled at the 5′ termini by using [γ-^{32}P]ATP (Amersham; >5,000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and polynucleotide kinase (42) as described (37). The labeled fragment was separated on a 5% polyacrylamide gel into leftward- and rightward-transcribed strands as described (43). After hybridization, the hybrid was treated with nuclease S1 as described (41) at 42°C for 30 min. The reaction products were analyzed on an 8% polyacrylamide sequence analysis gel (43). As a control, the rightward-transcribed strand was also used in hybridization experiments (data not shown).

RESULTS

Expression of the E2a Region of Ad2 DNA on Microinjection into Nuclei of *Xenopus laevis* Oocytes. Detailed analysis of the patterns of integration of viral DNA in Ad2-transformed hamster cell lines showed that cell lines HE1, HE2, and HE3 (24, 44, 45) contain the structural gene for Ad2-specific DBP (24). The DBP is encoded in the E2a region on the leftward-transcribed strand of the viral genome (Fig. 1). As pointed out previously, although the gene encoding the DBP is present in all three cell lines, the protein is expressed in cell line HE1 but not in cell lines HE2 and HE3 (10, 44, 46). All *Hpa* II sites in the E2a region in cell lines HE2 and HE3 are methylated at the internal cytosine (5′-C-C*G-G-3′) whereas, in cell line HE1, these sites are unmethylated (10).

An experiment was devised to decide whether DNA methylation was a consequence of lacking gene expression or was associated with the cause of gene inactivation. For the experiments, the E2a region of Ad2 DNA was cloned in the bacterial plasmid pBR322 for use as a viral test gene. The cloned *Hin*dIII A fragment of Ad2 DNA was methylated *in vitro* by using *Hpa* II DNA methyltransferase or was left unmethylated. Subsequently, methylated or unmethylated DNA fragments were microinjected into the nuclei of *Xenopus* oocytes.

It was ascertained that the injected viral DNA remained methylated over the 24-hr period of the experiment. Viral gene expression was then monitored by testing for the presence of Ad2-specific RNA.

It was important to show that *in vitro* methylation did not alter the conformation of the DNA. Plasmid pBR322 DNA was *in vitro* methylated by using *Hpa* II DNA methyltransferase or was left unmethylated. Subsequently, both DNA preparations were analyzed by electrophoresis on an agarose slab gel. The results (not shown) showed that the supercoiled DNA/open circular DNA ratio did not change after methylation. Thus, treatment with *Hpa* II DNA methyltransferase did not introduce nicks or breaks into supercoiled DNA.

DNA preparations were microinjected only if *in vitro* methylation had rendered the DNA completely refractory to digestion with *Hpa* II. As shown in Fig. 2, after extraction from *Xenopus* oocytes, the microinjected methylated Ad2 DNA segment was still sensitive to digestion with *Msp* I (Fig. 2, lane c) but was insensitive to *Hpa* II (Fig. 2, lane d)—i.e., the pattern of methylation had not changed over a 24 hr period. Unmethylated DNA remained unmethylated and could still be cleaved with either *Msp* I (Fig. 2, lane a) or *Hpa* II (Fig. 2, lane b).

Hpa II-methylated or unmethylated DNA was microinjected into nuclei of *Xenopus* oocytes: after 24 hr, the total intracellular RNA was extracted from the oocytes and analyzed for the presence of Ad2-specific sequences by gel electrophoresis, blotting, and DNA·RNA hybridization. Total Ad2 DNA, ^{32}P-labeled by nick-translation, was used as probe. It was apparent that the E2a region of Ad2 DNA was not expressed as RNA in *Xenopus* oocytes when the gene had been methylated at the *Hpa* II sites prior to microinjection (Fig. 3*A*, lanes a and c; Fig. 3*B*, lane b).

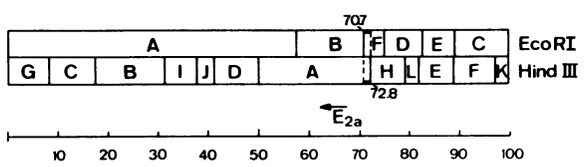

FIG. 1. Map of the E2a region of Ad2 DNA. The position of the subcloned fragment with map coordinates 70.7–72.8 that was used as a probe in some of our experiments is also indicated. The schemes represent the *Eco*RI and the *Hin*dIII maps of Ad2 DNA on a scale of fractional length.

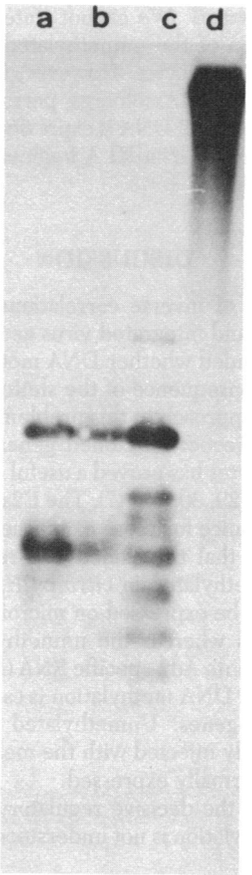

FIG. 2. Stability of the methylation pattern of the cloned *Hin*dIII A fragment of Ad2 DNA on microinjection into nuclei of *Xenopus* oocytes. The cloned DNA fragment was methylated *in vitro* (lanes c and d) by using *Hpa* II DNA methyltransferase (33, 34) or was left unmethylated (lanes a and b). The DNA preparations were then microinjected into nuclei of *Xenopus* oocytes and, 24 hr later, the total intracellular DNA was extracted. Subsequently, the DNA preparations were cleaved with *Msp* I (lanes a and c) or *Hpa* II (lanes b and d). The fragments were separated by electrophoresis on a 1.5% agarose slab gel, transferred to nitrocellulose filters, and visualized by hybridization to ^{32}P-labeled Ad2 DNA followed by autoradiography. Failure of cleavage by *Hpa* II indicated methylation at the internal cytosine of 5′-C-C-G-G-3′ sequences (35).

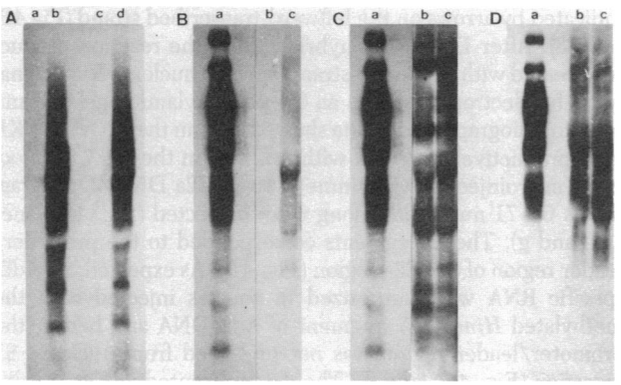

FIG. 3. Test for expression of methylated or unmethylated cloned *Hin*dIII A fragment of Ad2 DNA, of unmethylated h22 DNA (histone gene from sea urchin), or of pBR322 DNA after microinjection into oocytes from *Xenopus*. Twenty-four hours after microinjection, the total intracellular RNA was extracted and fractionated by electrophoresis on 0.8% agarose slab gels containing 2.2 M formaldehyde. The RNA was then transferred to nitrocellulose filters. (*A*) The cloned *Hin*dIII A fragment of Ad2 DNA was *in vitro* methylated (lanes a and c) by using *Hpa* II DNA methyltransferase or was left unmethylated (lanes b and d). The RNA blot was probed with ^{32}P-labeled Ad2 DNA. The RNA preparations analyzed in lanes c and d were treated with DNase. (*B*) Methylated cloned *Hin*dIII A fragment (lane b) or unmethylated DNA (lane c) was mixed with unmethylated recircularized h22 DNA before microinjection. RNA was analyzed as in *A*. *Hin*dIII-cleaved Ad2 DNA (lane a) was used as a size marker. (*C*) The blots obtained in *B* were rehybridized after autoradiography with ^{32}P-labeled h22 DNA from sea urchin and autoradiographed again. (*D*) *Xenopus* oocytes were microinjected with methylated (lane b) or unmethylated (lane c) pBR322 DNA devoid of viral DNA insertions. Twenty-four hours later, RNA was extracted, subjected to electrophoresis and transferred to filters, and probed with ^{32}P-labeled pBR322 DNA. *Hin*dIII-cleaved Ad2 DNA was used as an internal size marker (lane a). Viral DNA sequences were detected by hybridization to ^{32}P-labeled Ad2 DNA.

On the other hand, when the unmethylated cloned *Hin*dIII A fragment was injected, Ad2-specific RNA was readily made in abundance. In the latter case, the patterns of transcription were complicated due to the fact that the *Hin*dIII A fragment had not been excised prior to microinjection (Fig. 3*A*, lanes b and d; Fig. 3*B*, lane c). It was likely that transcription could start in the vector and thus render the results of transcription more complex.

The possibility existed that the inhibition of expression of Ad2 genes in *Xenopus* oocytes was due to a nonspecific factor affecting all transcription and acquired during the methylation reaction. To rule this out, unmethylated histone genes from sea urchin, h22 DNA, which were circularized (30), were microinjected together with the methylated cloned *Hin*dIII A fragment of Ad2 DNA. Twenty-four hours later, the RNA from the injected cells was extracted, analyzed by electrophoresis on 0.8% agarose slab gels containing ≈2.2 M formaldehyde (38), and transferred to nitrocellulose filters. By using the appropriate Ad2 DNA or h22 DNA probes, we showed that the Ad2 genes were not expressed when the *Hin*dIII A fragment had been methylated (Fig. 3*B*, lane b) while the h22 DNA was expressed as RNA (Fig. 3*C*, lane b). When unmethylated DNA was injected, both Ad2 DNA (Fig. 3*B*, lane c) and h22 DNA (Fig. 3*C*, lane c) were transcribed. These results showed that the block in expression of methylated Ad2 genes was not due to a hypothetical unspecific inhibitory factor in the preparation of the methylated Ad2 *Hin*dIII A clone. When the RNA blot from one of the experiments (Fig. 3*B*) was probed with ^{32}P-labeled pBR322 DNA, several pBR322 DNA-specific bands were observed in RNA extracted from oocytes that had been injected with unmethylated DNA. Only one plasmid-specific RNA band was seen when methylated DNA had been injected (data not shown). It is not known to what extent unspecific transcription started inside the unmethylated vector pBR322 DNA. When methylated or unmethylated pBR322 DNA without viral DNA inserts was injected into nuclei of *Xenopus* oocytes, no difference in the level of expression was apparent (Fig. 3*D*, lanes b and c). Apparently, the presence of an Ad2 promoter/leader region that could be recognized in a eukaryotic system influenced the (unspecific) expression of vector DNA sequences.

Specific Initiation of RNA Synthesis in the Cloned E2a Region of Ad2 DNA on Microinjection into *Xenopus laevis* Oocytes. The Ad2-specific RNA synthesized in *Xenopus* oocytes on microinjection of the cloned unmethylated *Hin*dIII A fragment of Ad2 DNA was analyzed for specific initiation. RNA isolated 24 hr after microinjection was hybridized to the leftward-transcribed strand of the 71.4–72.8 map-unit fragment of Ad2 DNA (Fig. 4*B*). This fragment had been terminally ^{32}P-labeled by using polynucleotide kinase and contained the late promoter/leader of the E2a region of Ad2 DNA (27, 28). In lytically infected cells, RNA synthesis is initiated at the nucleotides

indicated by arrows on the leftward-transcribed strand (Fig 4*B*; ref. 28). After DNA·RNA hybridization, the reaction product was treated with the single-strand-specific nuclease S1 and analyzed by electrophoresis on an 8% polyacrylamide gel (43) and by autoradiography. The data showed that, in the RNA from KB cells productively infected with Ad2 and in the RNA from oocytes microinjected with unmethylated E2a DNA, DNA fragments 69–71 nucleotides long were protected (Fig. 4*A*, lanes c, f, and g). These fragments corresponded to the promoter/leader region of the E2a region (Fig. 4*B*). As expected, no Ad2-specific RNA was synthesized in oocytes injected with the methylated *Hin*dIII A fragment of Ad2 DNA and hence, the promoter/leader region was not protected from nuclease S1 digestion (Fig. 4*A*, lane d). The data presented in Fig. 4 were obtained when the leftward-transcribed strand of the 71.4–72.8 map-unit fragment was used; the rightward-transcribed strand did not afford any protection. The results of a number of control experiments are also presented in Fig. 4*A*. Without the addition of RNA (lane b), without nuclease S1 treatment (lane a), or with RNA from oocytes that had not been microinjected (lane e), the promoter/leader fragment was not preserved. As markers, DNA fragments from a routine sequence analysis experiment (43) were used (lanes h and i).

We thus concluded that, on microinjection of the *in vitro*-methylated *Hin*dIII A fragment of Ad2 DNA into *Xenopus* oocytes, Ad2-specific RNA was not synthesized. When the unmethylated fragment was injected, Ad2-specific RNA was produced, and its synthesis was initiated at the same site(s) as in productively infected cells. In both productively infected KB cells and microinjected oocytes, RNA synthesis was on the leftward-transcribed strand. We cannot state with certainty that complete expression of the unmethylated E2a region of Ad2 DNA was attained in oocytes. However, it was demonstrated independently (F. A. M. Asselbergs, personal communication) that the E2a region of Ad2 DNA is expressed as the Ad2-specific DBP after injecting the *Hin*dIII A fragment of Ad2 DNA into *Xenopus laevis* oocytes.

DISCUSSION

The establishment of inverse correlations between levels of DNA methylation and integrated virus gene expression leaves the question undecided whether DNA methylation constitutes the cause or the consequence of the shutoff of specific genes. For a more direct approach to this problem, an *in vitro* system was used. Microinjection of cloned genes into the nuclei of *Xenopus laevis* oocytes has proved a useful system for the study of gene expression (29, 30, 36, 47). The E2a region of Ad2 DNA appeared a good choice for these experiments (10).

The observation that the cloned E2a region of Ad2 DNA, which had been methylated *in vitro* by *Hpa* II DNA methyltransferase, cannot be expressed on microinjection into nuclei of *Xenopus* oocytes whereas the unmethylated viral gene is readily transcribed into Ad2-specific RNA (Fig. 3) is compatible with the notion that DNA methylation is causally related to the shutoff of specific genes. Unmethylated sea urchin histone genes simultaneously injected with the methylated E2a region of Ad2 DNA are normally expressed.

The structure of the decisive regulatory sites that are sensitive to DNA methylation is not understood. It was interesting

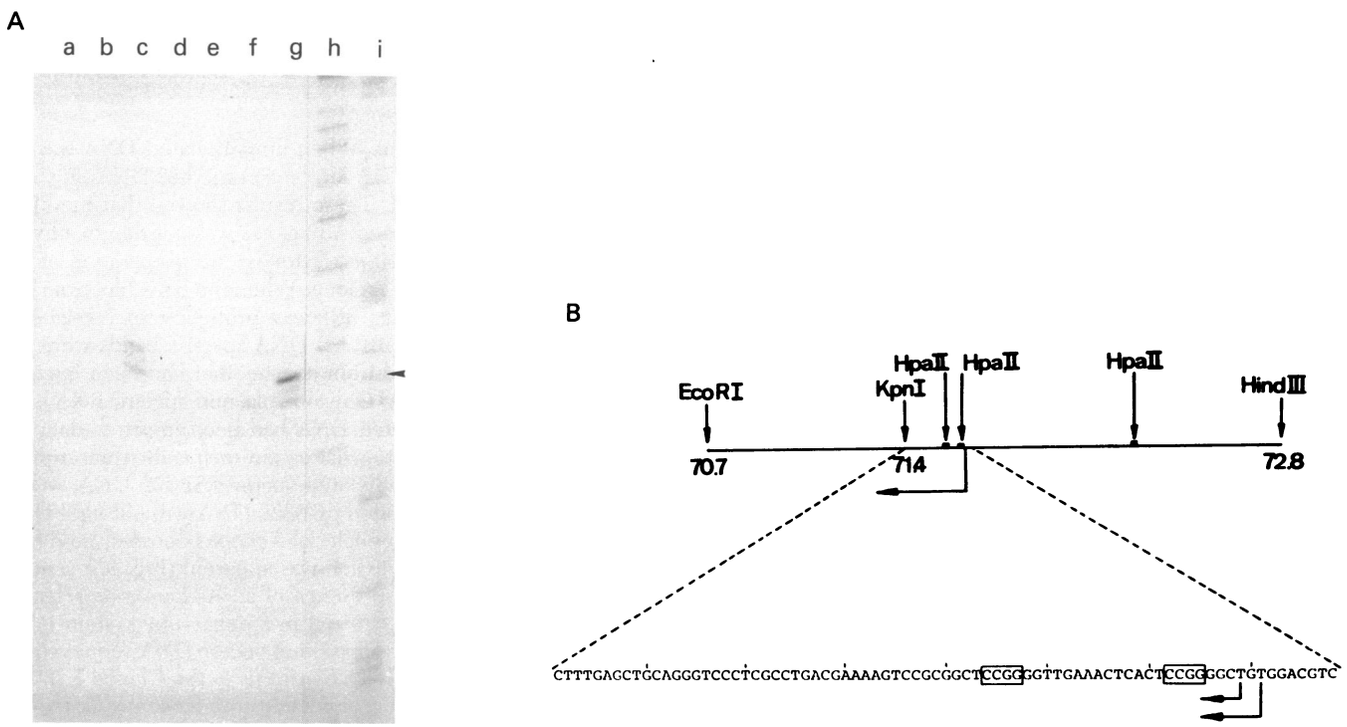

FIG. 4. (*A*) Specific initiation of Ad2-specific RNA from the E2a region in KB cells productively infected with Ad2 and in *Xenopus laevis* oocytes microinjected with the *Hin*dIII A fragment of Ad2 DNA. RNAs were hybridized to the ^{32}P-labeled leftward-transcribed strand of a 71.4–72.8 map-unit fragment of Ad2 DNA. Then, the hybrids were treated with nuclease S1, and the reaction products were analyzed. Lanes: a, nuclear RNA from KB cells isolated 12 hr after infection with Ad2 (the hybrids were not treated with nuclease S1); b, no RNA; c, total RNA extracted from *Xenopus* oocytes that had been microinjected with unmethylated cloned *Hin*dIII A fragment of Ad2 DNA; d, total RNA from oocytes that had been microinjected with *Hpa* II methylase-treated cloned *Hin*dIII A fragment of Ad2 DNA; e, RNA from oocytes that had not been microinjected; f, nuclear RNA isolated 12 hr after infection of KB cells with Ad2; g, cytoplasmic RNA isolated 12 hr after infection of KB cells with Ad2; h and i, size markers from a routine sequence analysis experiment (43). ◄, Seventy-nucleotide fragment. (*B*) Structure of the 71.4 (*Kpn* I site)–72.8 (*Hin*dIII site) map-unit fragment of Ad2 DNA, including an enlargement of the initiation site of the E2a region exhibiting part of the nucleotide sequence (28).

that methylation of the *Hin*dIII A fragment of Ad2 DNA by *Bsu*RI methylase did not inactivate the DBP gene on microinjection into *Xenopus* oocytes (unpublished result). *Bsu*RI methylase modified the 5′-G-G-C-C-3′ sequence. Thus, there was evidence that the methyl group had to be attached at a highly specific site to inactivate genes. It appears unlikely that a tetranucleotide in the methylated or unmethylated configuration by itself would be able to exert such crucial effects. Perhaps, a sequence of higher complexity encompassing one or several 5′-CpG-3′ sites constitutes the regulatory signal. These sites may merely have to permit the formation of specific secondary structures in DNA or DNA–protein complexes. These structures could then be stabilized by methylation of specific 5′-CpG-3′ doublets or methylation at highly specific sites might facilitate the stabilizing interactions of proteins with regulatory sequences of specific secondary structure. In this context, recent results (48, 49) on the structure of methylated versus unmethylated synthetic polynucleotides appear to be of interest. Apparently, methylated DNA has a much higher propensity to assume the left-handed Z configuration than unmethylated DNA, which remains in the right-handed *B* configuration. The availability of antibodies against Z-form DNA (50) will facilitate examination of the distribution of Z DNA in chromatin and correlate its distribution with that of transcriptionally active and inactive regions.

Another unresolved question pertains to the exact location of regulatory sites sensitive to DNA methylation. Are they located inside the structural gene, in the promoter/leader region, or upstream from either site in a more remote control region? These locations and the methylated sequences involved could be different in different genes. This notion would explain why inverse correlations between DNA methylation at a specific sequence and gene expression were not always perfect. The data accumulated so far will have to be refined by carrying out *in vitro* reconstructions in which certain parts of a gene or of its control regions will be methylated or left unmethylated prior to analysis in *in vitro* systems.

We are grateful to U. Günthert and T. Trautner, Berlin, for a gift of *Bsu*RI DNA methyltransferase. We thank D. Renz for providing valuable help in the cloning experiments and A. Weber for typing this manuscript. This research was supported by grants from the Ministry of Science and Research of the State of Northrhine-Westfalia (IIB5-FA8381) and by the Deutsche Forschungsgemeinschaft through Grant SFB 74.

1. Lindahl, T. (1981) *Nature (London)* **290**, 363–364.
2. Razin, A. & Riggs, A. D. (1980) *Science* **210**, 604–610.
3. Ehrlich, M. & Wang, R.Y.-H. (1981) *Science* **212**, 1350–1357.
4. Doerfler, W. (1981) *J. Gen. Virol.* **57**, 1–20.
5. Hattman, S. (1981) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York, London), Vol. 14, pp. 517–547.
6. Sutter, D., Westphal, M. & Doerfler, W. (1978) *Cell* **14**, 569–585.
7. Sutter, D. & Doerfler, W. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **44**, 565–568.
8. Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
9. Desrosiers, R. C., Mulder, C. & Fleckenstein, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3839–3843.
10. Vardimon, L., Neumann, R., Kuhlmann, I., Sutter, D. & Doerfler, W. (1980) *Nucleic Acids Res.* **8**, 2461–2473.
11. Cohen, J. C. (1980) *Cell* **19**, 653–662.
12. Guntaka, R. V., Rao, P. Y., Mitsialis, S. A. & Katz, R. (1980) *J. Virol.* **34**, 569–572.
13. Groudine, M., Eisenman, R. & Weintraub, H. (1981) *Nature (London)* **292**, 311–317.
14. van der Ploegh, L. H. T., Groffen, J. & Flavell, R. A. (1980) *Nucleic Acids Res.* **8**, 4563–4574.
15. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 4631–4641.
16. Sager, R. & Kitchin, R. (1975) *Science* **189**, 426–433.
17. Burton, W. G., Grabowy, C. T. & Sager, R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1390–1394.
18. Pollack, Y., Stein, R., Razin, A. & Cedar, H. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 6463–6467.
19. Wigler, M. Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C. & Axel, R. (1977) *Cell* **11**, 223–232.
20. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **7**, 2081–2090.
21. Constantinides, P. G., Jones, P. A. & Gevers, W. (1977) *Nature (London)* **267**, 364–366.
22. Taylor, S. M. & Jones, P. A. (1979) *Cell* **17**, 771–779.
23. Vardimon, L. & Doerfler, W. (1980) *Virology* **101**, 72–80.
24. Vardimon, L. & Doerfler, W. (1981) *J. Mol. Biol.* **147**, 227–246.
25. Bolivar, F., Rodriguez, R., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977) *Gene* **2**, 95–113.
26. Deuring, R., Winterhoff, U., Tamanoi, F., Stabel, S. & Doerfler, W. (1981) *Nature (London)* **293**, 81–84.
27. Chow, L. T., Broker, T. R. & Lewis, J. B. (1979) *J. Mol. Biol.* **134**, 265–303.
28. Baker, C. C. & Ziff, E. B. (1981) *J. Mol. Biol.* **149**, 189–221.
29. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1432–1436.
30. Telford, J. L., Kressmann, A., Koski, R. A., Grosschedl, R., Müller, F., Clarkson, S. G. & Birnstiel, M. L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2590–2594.
31. Rigby, P. J. W., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
32. Tjia, S. T., Carstens, E. B. & Doerfler, W. (1979) *Virology* **99**, 399–409.
33. Mann, M. B. & Smith, H. O. (1977) *Nucleic Acids Res.* **4**, 4211–4221.
34. Quint, A. & Cedar, H. (1981) *Nucleic Acids Res.* **9**, 633–646.
35. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 3231–3236.
36. Kressmann, A. & Birnstiel, M. L. (1980) *Transfer of Cell Constituents into Eukaryotic Cells* (NATO Advanced Study Institute), Ser. **31** (Plenum, New York, London), pp. 383–407.
37. Doerfler, W., Stabel, S., Ibelgaufts, H., Sutter, D., Neumann, R., Groneberg, J., Scheidtmann, K. H., Deuring, R. & Winterhoff, U. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **44**, 551–564.
38. Schirm, S. & Doerfler, W. (1981) *J. Virol.* **39**, 694–702.
39. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
40. Zimmermann, S. B. & Sandeen, G. (1966) *Anal. Biochem.* **14**, 269–277.
41. Berk, A. J. & Sharp, P. A. (1977) *Cell* **12**, 721–732.
42. Richardson, C. C. (1965) *Proc. Natl. Acad. Sci. USA* **54**, 158–165.
43. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
44. Johansson, K., Persson, H., Lewis, A. M., Pettersson, U., Tibbetts, C. & Philipson, L. (1978) *J. Virol.* **27**, 628–639.
45. Cook, J. L. & Lewis, A. M. (1979) *Cancer Res.* **39**, 1455–1461.
46. Esche, H. (1982) *J. Virol.* **41**.
47. McKnight, S. L., Gavis, E. R., Kingsbury, R. & Axel, R. (1981) *Cell* **25**, 385–398.
48. Behe, M. & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1619–1623.
49. Behe, M., Zimmermann, S. & Felsenfeld, G. (1981) *Nature (London)* **293**, 233–235.
50. Lafer, E. M., Möller, A., Nordheim, A., Stollar, B. D. & Rich, A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3546–3550.